Separation of enantiomers by capillary electrochromatography

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The current popularity of capillary electrochromatography (CEC) has led to an increasing number of studies on the development and evaluation of enantioselective CEC systems. These studies clearly demonstrate that the most prominent advantage of electrically driven separation methods, the vastly increased column efficiency as compared to pressure-driven chromatography, can also be experimentally achieved for the separations of enantiomers. In analogy to high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), several approaches have been used. The addition of a chiral selector to the mobile phase is the simplest method. Less erroneous and more elegant approaches are those that use open-tubular, conventional packed, and monolithic columns containing chiral stationary phases that stereoselectively interact with enantiomers. This review evaluates the new techniques and compares them to enantioselective HPLC and CE. Further, it describes the various concepts of enantioselective CEC and focuses on the current 'state-of-the-art' column technology. ©2000 Published by Elsevier Science B.V.

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1. Introduction

In many respects enantiomers have to be dealt with as different molecular entities. This applies in particular to the enantiomers of biologically active compounds like drugs, agrochemicals, pheromones, food additives, and flavors, which may have different activity and transformation profiles [1]. As a consequence, stereochemical aspects are routinely investigated in research and development and many of the mentioned compounds are marketed in enantiomerically pure form. To carry out stereoselective analyses, we rely on analytical separation systems with high separation power that allow the accurate determination of impurities amounting to less than 1% of the enantiomer, which is a widely accepted criterion of purity for enantiomeric compounds. The accurate determination of extreme enantiomer ratios like 99.9:0.1% is more challenging than the 'simple' separation of racemates. It could be greatly facilitated by enantioselective separation systems that simultaneously provide reasonable enantioselectivity, high efficiency, enhanced sample-loading capacity, and that allow the on-line coupling of highly sensitive, specific and universal detection methods like mass spectrometric (MS) detection. In theory, enantioselective capillary electrochromatography (CEC), which utilizes the molecular recognition principles well-known from liquid chromatography, and an electric field gradient as the driving force for analyte transport, meets these requirements. Therefore, CEC has a great likelihood of finding a place in the group of modern analytical enantioseparation techniques, including enantioselective capillary gas chromatography (GC) [2], supercritical fluid chromatography (SFC) [3], high-performance liquid chromatography (HPLC) [4], capillary electrophoresis (CE) and micellar electrokinetic chromatography (MEKC) [5], which are all implemented as standard techniques and are well described in the literature.

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2. Basic principles of CEC

Briefly, CEC can be described as hybrid method combining features of HPLC and CE. Accordingly, it involves the following processes, which are discussed in more detail in recent reviews on this topic [6]:

Analyte transport through the capillary column is primarily due to electroosmotic flow (EOF) (electroosmotic process). When an electric field is applied across the column, EOF originates from the electrical double layer at the charged solid–liquid interface of the stationary phase. The linear flow rate is proportional to the ζ-potential, which characterizes the double layer, and depends also on mobile phase properties (dielectric constant ε, viscosity η, ionic strength) as well as on the applied electric field strength E (Eq. 1). In contrast to HPLC and as in CE, two flow directions are possible: negatively charged surfaces show EOF from the anode to the cathode (cathodic EOF) and positively charged surfaces have EOF from the cathode to the anode (anodic EOF).

$$\mu_{eo} = -\frac{\varepsilon}{\eta}\zeta E \quad (1)$$

In addition to transport with the EOF, charged selectands (SAs) may have electrophoretic migration with the direction determined by the charge of the ion (electrophoretic process). The magnitude of the electrophoretic velocity u_{ep} is given by the relationship

$$\mu_{ep} = \frac{ze}{6\pi\eta r}E = \mu E \quad (2)$$

where z is the charge number, e the charge of an electron in Coulomb, r the ion radius, and μ the electrophoretic mobility. For weak electrolytes the effective electrophoretic mobility μ_{eff}, but not the mobility of the totally ionized species μ determines the electrophoretic velocity. It is related to μ by the degree of dissociation α: $\mu_{eff}=\mu\,\alpha$.

In pressure-assisted CEC, an additional pressure gradient is applied and the additional pressurized flow increment u_{press} may accelerate the analysis.

Overall, the apparent mean linear flow velocity of the analyte u_{app} is additively composed of the incremental transport processes:

$$u_{app} = u_{eo} + u_{ep} + u_{press} \quad (3)$$

Selectivity in CEC enantioseparation is always based on enantioselective molecular interactions between the analyte and the chiral stationary phase (CSP) or the pseudo-stationary phase (chiral additive) (chromatographic process), while the electrophoretic migration contributes just to overall analyte transport, since enantiomers have identical electrophoretic mobility.

Overall, the elution time t_e thus depends on the column length L, the chromatographic retention factor k, and the apparent mean linear flow velocity [7]:

$$t_e = L(1+k)/u_{app} \quad (4)$$

As a consequence of the distinctive electroosmotic and electrophoretic transport vectors, which may be co- or counter-directional and have a different actual magnitude, we can distinguish between several elution modes (Fig. 1A) (for the sake of simplicity, pressurized flow is not considered in this scheme). These modes of separation should be kept in mind in the further discussion.

3. Evaluation of enantioselective CEC in comparison to enantioselective HPLC and CE

From a practical point of view, what are the possible advantages of utilizing CEC instead of HPLC or CE/MEKC for separation problems? The miniaturized capillary format reduces the costs of operation compared to standard HPLC, but this could be realized also by capillary HPLC. The higher efficiency of CEC compared to pressure-driven chromatography, which is a result of less flow dispersion of EOF and occasionally of favorable pore flow, is without doubt the main impetus for CEC, and significantly improves the separation power as opposed to HPLC. Improved performance has been experimentally realized in almost all studies, even though capillary column packing and frit technology may not yet have been optimally developed. Through the multiplied peak capacity, a gain in analytical information can be obtained. Certainly, through orthogonality to HPLC and CE with regard to the separation mechanism, CEC is a valuable tool per se, and a gain for the analytical chemist.

In conclusion, aside from system stability and run-to-run as well as column-to-column reproduci-

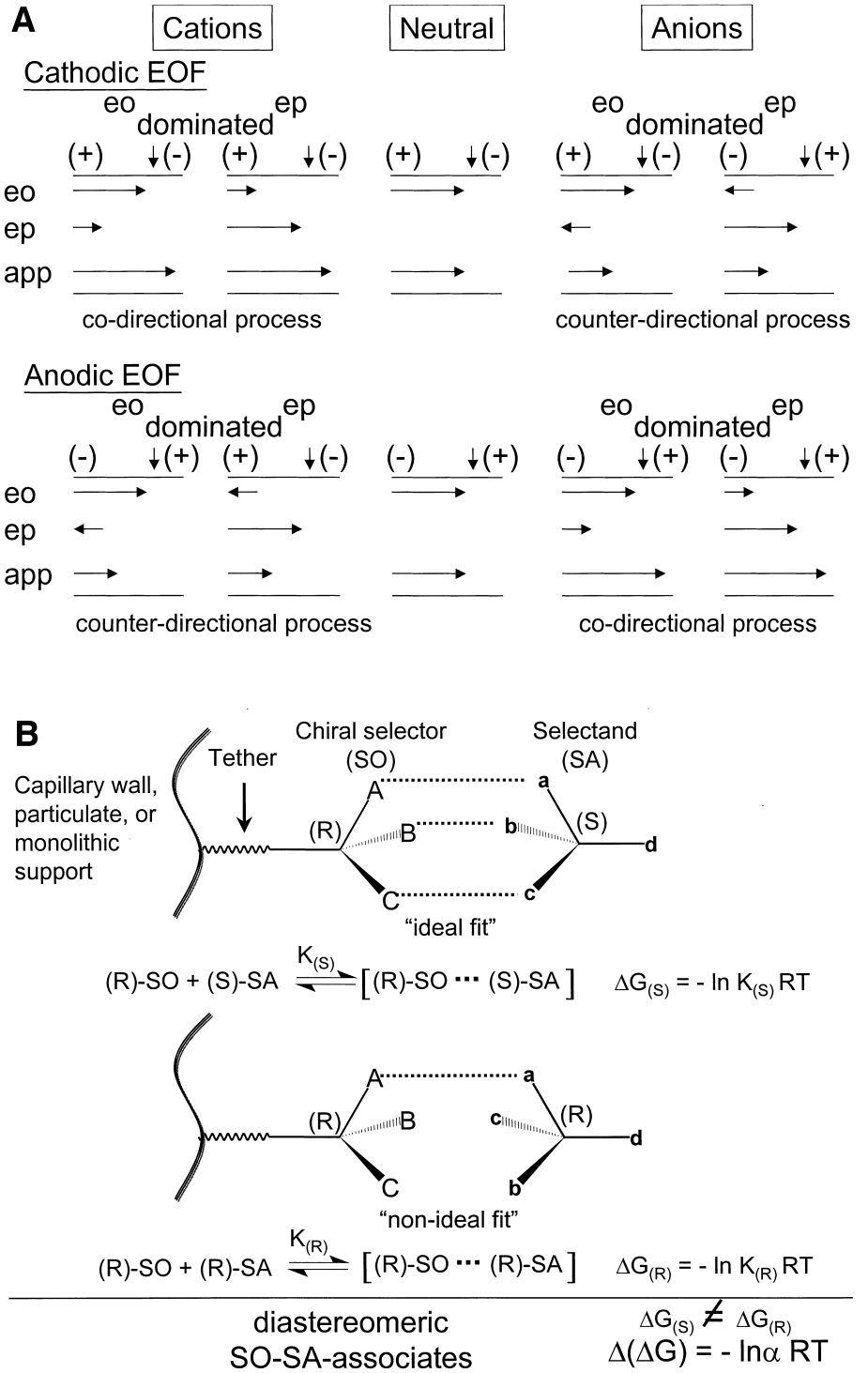

Fig. 1. A: Transport vectors of the CEC separations of cationic, neutral, and anionic analytes on negatively charged stationary phases (cathodic EOF) and positively charged stationary phases (anodic EOF) (for simplicity, the stationary phase is omitted from the scheme and pressurized flow is not considered). The arrow perpendicular to the capillary wall indicates the point of detection. Note also the changes from positive polarity mode (cathode at detection end) to the negative polarity mode (anode at the detection end), and vice versa. (eo: electroosmotic velocity, ep: electrophoretic velocity, app: apparent velocity). B: Concept of chiral recognition and direct enantioseparation by CSPs. Separation factor (α) in this context refers to the intrinsic enantioselectivity while the apparent separation factor calculated from chromatograms may be different for charged SAs due to their electrophoretic mobility.

bility concerns – and these aspects may improve in the future – we consider CEC to be more powerful than HPLC.

But how does CEC compare to CE/MEKC, which are becoming increasingly important for the separation of enantiomers in quality control, research, and even pharmacological studies? The almost unlimited flexibility to adjust the selectivity by stationary phase manipulation is a striking advantage of chromatographic methods over CE, and this would certainly also hold for CEC. However, the argument is weakened as CE enantioseparation involves pseudo-chromatographic principles, also allowing selectivity to be adjusted by structural variation of chiral selectors that are required to achieve resolution of enantiomers. Nevertheless, it appears that in CEC the same level of enantioselectivity can be preserved as in HPLC, while using analogous soluble selectors in CE often yields lower selectivity factors. (However, it should be noted that it is problematic to compare selectivity factors of these techniques, since the selectivity factors of charged analytes both in CE and CEC, which are usually directly calculated from the separation, are apparent α values). Lower enantioselectivity of these electrophoretic methods, however, may often be compensated by even higher efficiencies of CE/MEKC compared to CEC.

Moreover, one of the great advantages of CE is the feasibility and flexibility of incorporating the chiral selector to the background electrolyte. During method development, a variety of different selectors can be tested very easily, and even a combination of selectors with complementary chiral recognition capabilities can be added to the buffer solution. The use of such dual or multiple selector systems has proven to be advantageous for the separation of multiple racemates in a single run. On the other hand, the addition of the selector to the running buffer is certainly also one of the main drawbacks of CE/MEKC. Good solubility of the chiral selector in the running buffer is a prerequisite. This is often a critical point and limited, in particular in the past, the application of CE to the separation of enantiomers. Since the introduction of non-aqueous CE, the scope of enantioselective CE has been extended substantially. Unfortunately, to guarantee useful run-to-run reproducibility, the electrolyte solution has to be exchanged after each run and washing steps have to be inserted between the runs. This is connected with loss of the precious chiral selector and increasing analysis time.

Further, through the presence of the chiral selector and/or micelle-forming agent in the effluent, detection problems may easily arise. This holds also for CEC in the additive mode. UV detection is standard in CE. Generally, in the conventional setup, i.e. if the complete length of the capillary is filled with the selector buffer solution, the analytes are detected as diastereomeric SO–SA species. Due to the possibility of different absorption coefficients of diastereomeric species, detector responses for (*R*)- and (*S*)-enantiomers of the analytes may be significantly different. In particular strongly UV-absorbing selectors are prone to this effect, and additionally cause limitations in sensitivity. Even more pronounced is the probability of a different detector response for fluorescence detection (e.g. laser-induced fluorescence detection) that is frequently used in CE. In MS detection, interferences in the spectrum and pollution of the interface limit MS compatibility. These detection problems in enantioselective CE caused by selectors in the running buffer have to be circumvented by applying the partial filling technique. In sharp contrast, enantioselective CEC using the CSP mode eliminates the problems associated with the selector in the running electrolyte and experiences no limitations whatsoever with regard to detection. Therefore, it is really advantageous in this respect, giving full MS compatibility.

Another strong argument in favor of CEC is its higher sample-loading capacity compared to CE. Through the large adsorption surface that is introduced by the stationary phase, the sample capacity is dramatically increased. This is an issue, because substantial amounts of sample have to be injected to reach the detection limits of trace amounts of enantiomeric impurities (often $< 0.1\%$). Overlapping of a minor component with the major one may easily occur if the resolution is not high enough and the system is overloaded. In this context, it should also be pointed out that on-column sample preconcentration can be exploited very easily in CEC to improve the limits of detection and quantitation.

Considering all these factors, we regard enantioselective CEC as a very powerful technique, in particular when coupled with MS detection. The optimal compatibility of CEC–MS coupling could be a main driving force for the acceptance of this new separation technique as a complement to enantioselective HPLC and CE. Before CEC replaces HPLC and CE more broadly, however, further improvements in column technologies, system stabilities,

and column-to-column reproducibilities have to be demonstrated, and the commercialization of a variety of enantioselective CEC columns at reasonable prices will be required.

4. General concepts of CEC enantioseparations

4.1. Indirect enantioseparation and separation of diastereomers

Although the indirect enantioseparation concept is not yet very common in CEC, it is worth noting. First, the analyte (the SA) is derivatized with an enantiomerically pure (highly enriched) chiral reagent, to form a pair of diastereomers that can be separated using an achiral stationary phase and non-enantioselective CEC system. The diastereomers are separated due to differential adsorption onto the stationary phase. In case of charged diastereomers, different electrophoretic migration may also contribute to the overall separation process.

The indirect approach is not very popular although it utilizes commercially available reversed phase (RP) CEC columns which have proven to exhibit quite high column efficiencies in RP CEC. A possible explanation for the poor popularity of the indirect method could be its susceptibility to errors related to quantitative analysis, such as those caused by enantiomeric impurities in the chiral derivatization agents, different detector response for both diastereomers, kinetic resolution in the course of incomplete derivatization, and racemization under derivatization conditions. Therefore, this method requires careful validation to avoid these errors [8]. In addition, electrokinetic injection in CEC is an additional source of discrimination between the diastereomers, e.g. in the case of charged diastereomeric molecules, resulting in changes of peak area ratios and requires corrections. The feasibility of the method, i.e. of CEC separations of diastereomers, was shown by Meyring et al., who demonstrated the CEC separation of (*R*)-(+)-thalidomide metabolites [9].

4.2. Direct enantioseparation

Two different approaches exist for direct CEC enantioseparation: (i) the chiral selector (SO) is added to the mobile phase (additive mode), or (ii) the selector is immobilized onto a solid support or the capillary wall affording a CSP. The concept of direct enantiodiscrimination is schematically outlined in Fig. 1B and represents the basis of all further discussed enantiomer separation technologies applicable in CEC.

4.2.1. Use of chiral additives

The additive mode is a simple option to achieve enantiomer separations using commercially available achiral, typically standard RP columns. In this case, SO–SA interaction takes place in the mobile phase and/or the chiral selector molecules may adsorb onto the RP phase, thus generating a dynamic coating controlled by secondary equilibria. The small internal volume of the capillary column makes this technique attractive and affordable since selector consumption is low.

While this approach is very simple from the practical standpoint, the separation mechanism in this technique is rather complex. The separation of charged SAs further adds to the complexity, since both electrophoretic and chromatographic mechanisms are involved. Enantioselectivity is controlled by a number of factors: (i) the difference in the equilibrium constants for complex formation of (*R*)- and (*S*)-enantiomers with the chiral selector (intrinsic selectivity); (ii) the concentration of the selector at equilibrium that (in addition to the partition coefficient of the selector) also determines the selector density adsorbed onto the RP material; (iii) the differential partitioning of the analyte enantiomers due to formation of diastereomeric complexes (chromatographic contribution); and, to some extent, (iv) the difference in mobility of free and complexed enantiomers [7]. The situation might be more difficult in cases where the EOF and electrophoretic migration of charged analytes have opposite directions. Thus, as a consequence of different contributions of these two processes, reversal of elution order may occur under different mobile phase conditions, although the relative intrinsic affinities of the enantiomers to the selector remain the same. This effect is well-known in CE enantioseparation [5].

The most popular chiral additives are native α-, β-, and γ-cyclodextrins and their neutral, positively, and negatively charged derivatives. For example, neutral chlorthalidone enantiomers were separated on a CEC column packed with 3 μm ODS using hydroxypropyl-β-cyclodextrin as a chiral selector [10]. Wei et al. described the separation of phenyl-

ephrine and synephrine enantiomers on a bare silica stationary phase using hydroxypropyl-β-cyclodextrin as a chiral additive [11]. The enantioseparation of salsinol by CEC using columns packed with an ODS stationary phase and β-cyclodextrin as the chiral additive and 1-heptanesulfonic acid as an achiral ion-pairing agent has been reported by Deng et al. [7]. They thoroughly discuss the separation mechanism of CEC in the presence of chiral mobile phase additives. They were able to derive and experimentally validate a theoretical model for enantioselectivity in these systems which is applicable to both neutral and charged compounds.

Some other selectors have also been found useful as chiral additives. For example, a quinine carbamate was used in both aqueous and non-aqueous mobile phases as a chiral ion-pairing agent for the separation of chiral acids by CEC on capillary column packed with 3 μm ODS [12]. In this case, stereoselective ion-pair formation resulted in enantioseparation. High efficiencies of up to 160 000 plates/m were achieved in this countercurrent-like separation process using conditions under which the cathodic EOF towards the injection end of the capillary was minimized and electrophoretic transport dominated. Thus, the negatively charged analytes were eluted in the negative polarity mode. In contrast, low efficiency but higher enantioselectivity were afforded under conditions that resulted in electroosmotically dominated elution (positive polarity mode) with a minor electrophoretic contribution. Opposite elution orders were observed for the two distinct separation modes, which are a result of different contributions of oppositely directed electroosmotic and electrophoretic processes, although the relative intrinsic affinities of the enantiomers to the selector did not change.

In CEC, if the selector is incorporated in the mobile phase, the same problems and limitations as pointed out for CE, such as loss of the precious selector, detection problems, and the possibility of different detector responses for both enantiomers, exist (vide infra). Moreover, with the addition of the achiral stationary phase, the separation mechanism becomes more complicated compared to CE. Therefore, CEC enantioseparation with the selector as a buffer additive may not offer any real advantage over enantioselective CE.

4.2.2. Enantioselective stationary phases

The problems of the additive mode do not exist if the separations are carried out using CSPs with immobilized chiral selectors. The current literature presents three different approaches to capillary columns with CSPs:

1. Open-tubular (OT) columns with the chiral selector coated, adsorbed, or covalently bonded to the capillary wall. Successful separation requires capillary tubes with a narrow inner diameter (< 50 μm).
2. Columns packed with a stationary phase that has the selector covalently bonded or coated onto standard chromatographic supports. Typical supports are silica or alternatively polymer beads and the CSPs are packed into 75 or 100 μm i.d. fused-silica capillaries. These CSPs resemble those used in conventional HPLC.
3. Specifically designed monolithic chiral phases that are prepared in situ within the confines of a capillary and have selectors or chiral cavities incorporated into a continuous polymer matrix (monolith or rod) appear to be well suited for enantioselective CEC.

In all of these columns, the enantioselectivity (α) is directly related to the difference in the equilibrium constants of SO–SA complex formation of (*S*)- and (*R*)-SA, as schematically outlined in Fig. 1B. The applied electrical field is the driving force for analyte transport through the column. Neutral SAs are transported through the column by EOF while electrophoretic migration additionally contributes to the transport of charged molecules (see Fig. 1A). Once more, it should be emphasized that the vectors of electroosmotic and electrophoretic transport might have different directions. As a consequence of the movement of the sample zones of charged analytes relative to the flow marker induced by electrophoretic migration, the calculated apparent enantioselectivity values may differ from the intrinsic enantioselectivity (as defined in Fig. 1B) that would be obtained in HPLC. In an extreme case, if the analytes elute before the flow (EOF) marker, the chromatographic terminology is less appropriate. For such separations with a significant or dominant electrophoretic transport contribution, the terminology of electrophoresis may be more suitable.

External pressure applied at one end of the capillary may accelerate the analysis. However, the effect of pressurized flow may negatively affect the plug-like flow profile typical of EOF-driven systems and give a parabolic profile with a concomitant decrease in column efficiency.

5. CEC separation with enantioselective stationary phases

Although there are a number of approaches, enantioselective CEC implementing CSPs with immobilized selectors is currently the most promising technique with respect to the column technology. Therefore, only these columns and separation media will be discussed in detail.

5.1. OT columns

In enantioselective OT columns, the chiral selector is immobilized onto the capillary wall by coating, covalent binding, or adsorption. Mostly, the residual silanol groups of the silica wall are the source for EOF, and therefore the flow may be rather poor in OT devices with a well coated surface. Obviously, there is no need for retaining frits, and very long capillaries can be prepared with homogeneous coating across the total length. The mobile phase can be exchanged very quickly, thus yielding shorter equilibration times. The only serious drawback of this approach is the low sample-loading capacity of the stationary phase as only a very small surface area is available for its bonding. This results in a rather unfavorable phase ratio. Therefore, these columns may easily be overloaded causing peak asymmetry and poor efficiency.

Several requirements have to be met to obtain OT CEC columns with high efficiencies [13–15]. Radial transport of the analyte within the mobile phase to the stationary phase at the wall occurs only by slow diffusion. Slow radial transport contributes to band spreading, particularly if the inner diameter of the capillary becomes larger and the flow velocity increases. The Golay equation also suggests that this contribution is more significant at higher retention factors. In addition, the efficiency is directly affected by the thickness of the stationary phase layer; thinner films afford higher numbers of theoretical plates.

Due to the limited sensitivity of on-column UV detection (the standard detection mode in most instruments), capillaries with 50 μm i.d. are typically used in OT CEC columns. They represent a trade-off between the requirement of high efficiencies typical of narrower tubing and the detection ability of the system. Vindevogel and Sandra concluded that wider capillaries should be operated at slower flow velocities using a mobile phase offering lower retention factors. However, OT electrochromatography (OT-EC) in such large diameter capillary columns performs at a 'suboptimal efficiency' compared to CE or MEKC [13].

The first enantiomer separations using OT-EC were demonstrated by Mayer and Schurig [16]. They used 80–100 cm × 50 μm i.d. fused-silica capillaries coated with a 0.2 μm film of Chirasil-Dex, a poly(dimethylsiloxane) with covalently bonded permethylated β-cyclodextrin (ca. 16 wt%). This phase was found to be stable under conditions involving 0.02 mol/l borate/phosphate buffer at pH 7. Although the silanols of the silica surface were, at least to a certain extent, shielded by the polysiloxane coating, a sufficient amount of these charged functionalities remained accessible to achieve satisfactory EOF velocities. Selectivity factors (α) of 1.0–1.2 were obtained for polar, neutral (1-phenylethanol) and charged chiral compounds (ibuprofen) that contain aromatic rings capable of inclusion into the hydrophobic cyclodextrin cavity. Due to an excellent efficiency of up to 300 000 theoretical plates/m, these columns enabled baseline separations. However, an increase in the film thickness from 0.2 to 0.4, and subsequently to 0.8 μm, resulted in a dramatic loss in efficiency from 30 500 to 24 000 to 9500 theoretical plates per column. With increasing film thickness, retention factors increased from 0.21 to 0.32 and 1.10, while selectivity factors remained almost unchanged (1.17, 1.19, and 1.12, respectively). The higher retention factors and the increasing contribution of resistance to mass transfer within the thick layer of the stationary phase were thought to be responsible for the rapid decrease in efficiency [13,17].

The Chirasil-Dex phase was originally developed for enantioseparations in GC mode. Later, this stationary phase was also applied in unified enantioselective chromatography using an OT capillary column in several chromatographic modes such as OT-GC, OT-SFC, OT liquid chromatography (OT-LC), and OT-EC (Fig. 2) with the option of an easy and reversible switching between these modes [14,18].

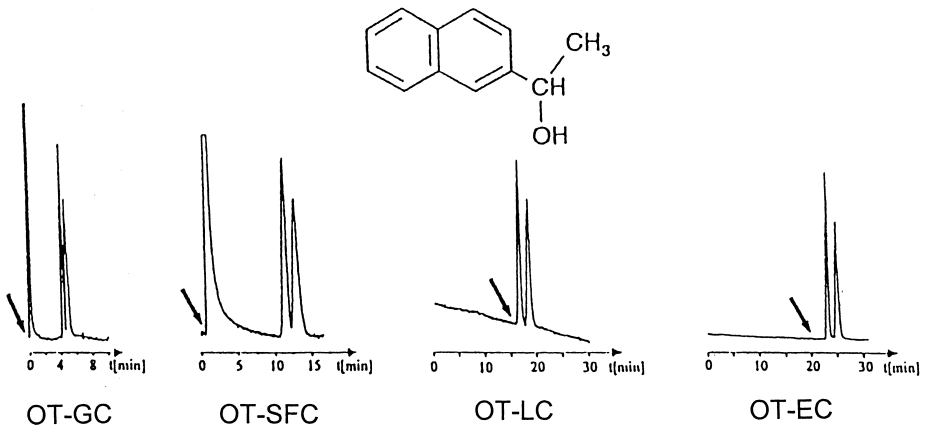

Fig. 2. 'Unified' enantioselective chromatography using a capillary column coated with Chiralsil-Dex (film thickness ~0.14 mm). OT-GC: T=120°C, p=1.1 bar (He); OT-SFC: p=68 bar (CO_2), T=55°C; OT-LC: p=0.14 bar, T=35°C, 20 m*M* phosphate buffer pH 7; OT-EC: 30 kV, 10 mmol/l borate/phosphate buffer (pH 7.5), T=60°C. The arrows indicate the approximate elution times for an unretained void marker (reprinted with permission from [14]. © 1999 Wiley-VCH).

Francotte and Jung demonstrated the feasibility of enantioselective OT-EC with 50 μm i.d. capillaries coated with tris(3,5-dimethylphenyl carbamoyl) cellulose and tris(*para*-methylbenzoyl) cellulose derivatives for a variety of chiral pharmaceuticals [15]. They found that a film thickness of only 0.025 μm led to the best separations with efficiencies 2–4 times higher than those found for OT-LC, while enantioselectivity remained unchanged. Capillaries coated with 0.009 μm thin films did not resolve the enantiomers due to overloading. In contrast, thicker films of 0.1 μm afforded sufficient enantioselectivity but efficiency decreased significantly. This is largely in agreement with the findings of Mayer and Schurig [17]. The surface coverage of the cellulose derivative coatings was very efficient – a film thickness of 0.009 μm reduced the EOF velocity dramatically. While 3 min elution time was observed for the void marker in an untreated fused-silica capillary, about 20 min was required for the capillaries with a film thickness of over 0.009 μm. In addition, the flow was irreproducible and limited column lifetime, mostly as a consequence of sudden clogging, was reported. All of these serious problems limited the practical applicability of these cellulose-coated OT columns.

In another approach, thin porous films of highly crosslinked molecularly imprinted polymers anchored to the inner walls of 25 μm i.d. fused-silica capillaries were prepared by an in situ polymerization process [19]. Imprinting involves the polymerization of functional monomers which are preorganized by non-covalent interactions around a template molecule that, after it is removed, leaves behind chiral cavities in the polymer with defined spatial shape and well arranged functionalities complementary to the target. These cavities enable re-binding of the template molecule and separation from its enantiomeric counterpart in the CEC separation process. Polymers imprinted towards *N*-dansyl (*S*)-phenylalanine were directly prepared in vinylized capillaries by copolymerization of methacrylic acid and 2-vinyl pyridine (functional monomers), with crosslinkers such as ethylene dimethacrylate (EDMA) or trimethylolpropane trimethacrylate (TRIM) in the presence of toluene and acetonitrile as porogenic solvents and the template molecules. Aprotic porogens are selected to avoid interferences with non-covalent interactions during imprinting. Typically, high crosslinker-to-monomer ratios of 5:1 for EDMA and 1:1 for TRIM were used to achieve rigidity of the three-dimensional structure required to maintain fidelity of the binding sites. After filling the capillary with the polymerization mixture, the polymerization process was thermally initiated. The preparation of the capillary column was completed by applying a vacuum at one end of the capillary and a pressure of 0.7 MPa at the other to remove the porogens and promote shrinkage of the polymer matrix to form a thin film. This method of film preparation was difficult and occasionally resulted in permanent occlusion if high

polymerization temperatures and long times were used. Scanning electron micrographs showed that the coating was not uniform. Despite non-uniformity, good enantioselectivity and a high efficiency of 248 600 plates/m for the non-imprinted enantiomer were obtained. However, the peak of the retained enantiomer was broad and only a modest efficiency of 8000 plates/m was achieved. This poor efficiency for the imprinted enantiomer is an intrinsic problem of imprinted phases for both liquid chromatography and electrochromatography and can be explained by the presence of polydisperse adsorption sites with different binding kinetics and only a limited number of highly selective imprints.

Effective control of EOF has been demonstrated by Sinibaldi et al. [20]. They coated 100 μm i.d. capillaries by a two-step procedure. First, a poly(vinyl siloxane) film was covalently bonded to the capillary wall, and then a semi-synthetic selector, ergoline alkaloid (+)-1-allyl-(5*R*,8*S*,10*R*)-terguride, was chemically bonded by a free radical addition reaction. As expected, within the pH range of 2.5–4, an anodic EOF that originated from the positively charged ergoline moieties was observed, favorable for the analysis of acidic compounds. Despite moderate enantioselectivity, high efficiencies of up to 150 000 plates/m resulted in baseline separations of dansylated amino acids and flobufen.

Another group of OT columns has covalently bonded well defined low and high molecular weight selectors. Compared to columns with coated polymeric selectors, columns of this type may have even lower selector-loadings and further reduced sample-loading capacities. Only a few studies have been reported. For example, bovine serum albumin was covalently attached by a gentle coupling reaction with tresyl-activated diol functionalities of a modified fused-silica capillary [21]. These capillary columns performed well in the separation of a number of DNP-amino acid and 3-hydroxy-1,4-benzodiazepine enantiomers.

Pesek et al. developed a method of etching the inner wall of 20–50 μm fused-silica capillaries with ammonium hydrogendifluoride followed by functionalization and bonding of the selector [22]. The etching process generates a rough surface and significantly increases the surface area thus enabling higher loading with ligands and higher sample capacity. Several types of selectors were attached via reactive hydride surface functionalities, including *N*-benzoyl-(*R*)-(+)-1-(α-naphthyl)ethylamine, a typical 'Pirkle type' selector. Although enantiomers of *N*-3,5-dinitrobenzoyl-alanine methyl ester could be separated, peaks with poor symmetry and low efficiency were obtained.

The strong adsorption of basic proteins, peptides, and amino acids, often encountered as an undesired side effect in the CE separation of these compounds, was utilized to generate OT columns with an adsorbed layer of lysozyme, cytochrome *c*, Lys-Tyr, Lys-Ser-Tyr, and Lys as chiral selectors [23,24]. The adsorption occurs on rinsing the capillary for 3–8 min with an aqueous solution of the selector. The simple preparation procedure allows the use of 10 μm i.d. capillaries that led to improved phase ratio and column efficiency. These capillaries had a lifetime of several weeks. Separations of the enantiomers of mephenytoin, aromatic amino acids, and both PTH and dansyl derivatives of amino acids with column efficiencies as high as 500 000 plates/m close to those of electrophoretic separations were reported. However, poor peak shape has often been observed, and adsorption of the selector at the detection window significantly impaired detector response.

Although the feasibility of enantiomer separation by electrochromatography with 'enantioselective OT columns' has been demonstrated and a number of successful separations have been reported, the low phase ratios and poor sample-loading capacity make them prone to overloading that negatively affects resolution. Overloading, in turn, may somehow be demanded due to the use of insufficiently sensitive detection systems. Overall, this approach appears to be less popular for enantioselective CEC.

5.2. Capillary columns packed with conventional CSPs

This type of enantioselective column mimics the standard HPLC column technology, and the chiral packings are essentially identical or very similar to those currently used in enantioselective HPLC. Smaller particles and/or longer columns can be utilized since back pressure limitations are not an issue in CEC. This together with the flat EOF profile enables the generation of higher numbers of theoretical plates per column in CEC applications compared to conventional HPLC. Both CSP chemistry and expertise are readily available from earlier developments in HPLC. Therefore, it currently appears to be more straightforward to adjust the

chiral HPLC packings to CEC requirements and work out capillary column packing protocols than to develop completely new concepts of dedicated chiral phases and columns thereof. As a result, these columns are very popular and are intensively investigated.

Particulate CSPs are packed from a slurry in solvents or supercritical fluids at high pressures of 30–100 MPa or electrokinetically into 50–100 μm i.d. capillaries. With the exception of specifically designed tapered capillary tubes, retaining frits must be fabricated at both the inlet and outlet ends to hold the packed bed in the capillary during the electrochromatographic process. This multistep procedure often has a number of difficulties. The preparation of capillary columns with good stability, permeability, and column-to-column reproducibility requires specialized skills. Often, good frits may not be directly sintered using the CSP. Therefore, other materials, such as native silica beads, are used for the frit fabrication [10,25]. The preparation of CEC columns has improved during the last few years, and, although still laborious, no longer limits the production of capillary columns with good chromatographic performance. Consequently, a wide variety of particulate CSPs, almost exclusively modified silica beads, have been packed in capillaries and successfully used in CEC.

Three operational modes can be distinguished in HPLC enantioseparation using conventional chiral packings: normal-phase, polar organic, and reversed phase. The last two modes are capable of separating charged molecules. CSPs that are run in the RP mode (with buffered hydro-organic mobile phases) or in the polar organic mode (with non-aqueous mobile phases containing polar organic solvents and organic acids and bases) are the first choice for CEC application. These conditions can often be directly transferred to CEC without a concomitant loss in enantioselectivity. Due to a favorable dielectric constant/viscosity ratio, acetonitrile as organic modifier is beneficial in achieving high EOF velocities and is therefore preferred over other solvents like methanol.

CSPs based on proteins, macrocyclic selectors (cyclodextrins, macrocyclic antibiotics), and anion exchangers such as those with attached quinine and quinidine derivatives are well suited for CEC, since they typically operate under RP conditions. The high enantioselectivity levels of HPLC can usually also be maintained in CEC. These chiral selectors involve either of the following interactions, that are fully effective in the aqueous mode, as primary driving forces for SO–SA complex formation:

1. inclusion into a chiral cavity or cleft by hydrophobic interactions, typical of cyclodextrins and macrocyclic antibiotics;
2. strong ion–ion interactions found for proteins and ion exchangers, and
3. simultaneous, multiple hydrogen bonding typical of macrocyclic antibiotics, proteins and crown ethers.

Effective enantioselective molecular recognition is achieved when these primary SO–SA interactions are accompanied by additional intermolecular forces such as hydrogen bonding, π–π interactions, van der Waals type interactions, and steric barriers that may facilitate or prevent an attraction.

Typical 'normal-phase' packings based on semisynthetic and synthetic polymeric selectors such as cellulose or amylose derivatives, chiral polyacrylamides and polymethacrylates, and donor–acceptor 'Pirkle type' selectors for which SO–SA complexation and stereoselective discrimination relies primarily on hydrogen bonding and π–π interactions are less effective in the RP mode. Therefore, these CSPs usually exhibit lower enantioselectivity under typical CEC conditions.

A suitable compromise between two counteracting effects has to be made to achieve a useful enantioseparation:

1. (relatively) strong SO–SA interactions that are the basis for selectively, and
2. sufficient elution speed to achieve the separation within a reasonable run time.

Therefore, the strength of the analyte–sorbent interactions must be controlled, typically by changing the composition of the mobile phase. This is not as trivial in CEC as in HPLC. In contrast to HPLC, tuning selectivity and/or balancing retention in CEC always affects the flow rate and, in the case of charged analytes, also their electrophoretic migration. This may lead to an opposite effect on run times. For example, ion–ion interactions are very strong and can be offset by an increase in the ionic strength of the buffer. However, such changes directly affect the EOF and the stability of the separation system since high current is produced in

solutions with a high ionic strength. Therefore, ionic interactions are most difficult to control in CEC. In most cases, mobile phases that afford high EOF, such as mixtures of acetonitrile, and low ionic strength buffer are used, even though it may not be optimal with regard to retention, enantioselectivity and efficiency. Although this may easily lead to failure of the initial separation attempts, the frames of experimental conditions that can be used in CEC are very much wider than currently exploited and than has become routine.

A strong and stable EOF is an important requirement in CEC. Besides the mobile phase, stationary phase parameters determine the ζ-potential under the working conditions and have influence on both the EOF velocity and direction as well. Provided that no EOF modifiers like cetyltrimethylammonium bromide are added to the mobile phase, the negatively charged residual silanol functionalities of silica-based CSPs with neutral chiral selectors, such as cellulose derivatives, synthetic polymers, cyclodextrins, and Pirkle type selectors, which normally do not contain any other charged groups, allow cathodic EOF. Non-endcapped CSPs with high levels of residual silanol groups are preferred. In contrast, selectors with charged functionalities such as proteins, macrocyclic antibiotics, and chiral anion exchangers may, depending on the p*K* values of the ionizable groups of the selector and pH of the mobile phase, significantly contribute to and even reverse the direction of the EOF. Whether there is a positive effect on the mass transfer kinetics, if the selector itself is the source for EOF as opposed to EOF being generated at the support, remains unclear and difficult to assess. Apparently, it is not detrimental when the EOF is generated directly at the interaction site (vide supra).

Generally, the selection of an appropriate support material for the preparation of CSPs to be used in CEC is more critical than in HPLC. Different silica materials may vary significantly in acidity and density of silanol groups. As a result, the EOF differences of CSPs prepared from different types of silica materials may be surprising. While 3 μm silica beads with 100 Å pore size are currently a standard for CEC, 5 and 7 μm beads with wide pores of 300–4000 Å also appear promising. They allow a convective flow through the pores that outweighs the negative effect of larger bead size on column efficiency, and in addition may yield much faster separations [26].

5.2.1. Proteins as selectors

The first enantiomer separations by CEC with packed capillaries have been reported by Li and Lloyd using a commercially available CSP, Chiral AGP – 5 μm HPLC packing, with attached α_1-acid glycoprotein (AGP) [27]. Frits were made by direct sintering of this CSP. About 45% of the protein consists of carbohydrate and sialic acid residues, respectively. This protein is acidic with a p*I* value of 2.7 possessing multiple charged sites. The negative net charge of AGP in the typical working pH range between 3 and 7.5 may contribute to the cathodic EOF of the CSP. Generally, the AGP phase can stereoselectively bind a number of acidic, basic, and neutral SAs. Hence, a number of pharmaceuticals such as β-blockers, barbiturates, benzoin, cyclophosphamide, and disopyramide, for which this CSP exhibited selectivity in HPLC, were also well separated in CEC using 2 mmol/l phosphate buffer (pH 6.8) with 2–15% of 2-propanol as organic modifier. Column efficiencies of up to 35 000 plates/m were achieved and were better than those found in HPLC. However, some acidic compounds could not be eluted from this CEC column. One explanation of this phenomenon is the electrophoretic migration opposite to EOF. Another argument takes into account the strong ionic interactions between oppositely charged analytes and binding sites of the protein that cannot be counterbalanced by the low ionic strength buffer required to avoid high current and bubble formation. The latter argument is supported by the fact that also some basic drugs could not be eluted from the column and that in HPLC much higher buffer concentrations, e.g. 10–100 mmol/l phosphate buffers, must be used to obtain sufficiently high elution strength.

Human serum albumin-based CSP was used for the separation of benzodiazepines (temazepam) and benzoin [28]. These results indicate that the immobilized proteins preserve their active conformation in the electric field and maintain their chiral recognition capability even in CEC mode.

5.2.2. Macrocyclic antibiotics

Macrocyclic antibiotics are another class of multiply charged selectors. Both vancomycin- [29,30] and teicoplanin-derived CSPs [25] were evaluated in CEC. Similar to the protein CSPs, these phases are not completely defined in terms of their bonded structure, since current immobilization procedures address multiple functionalities and may lead to

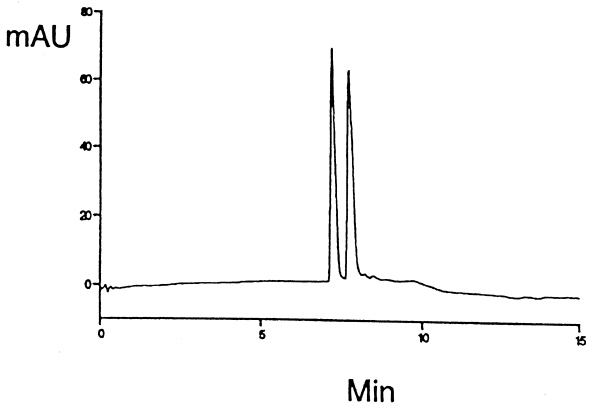

Fig. 3. CEC enantioseparation of metoprolol on vancomycin-based CSP in the polar organic phase mode. Mobile phase: MeOH/MeCN/AcOH/NEt_3 (50:50:0.1:0.3, v/v/v/v), capillary: 335 mm×75 μm i.d. (L_{eff} 265 mm), 10 kV, 15°C (reprinted with permission from [30]. © 2000 Elsevier).

selectors attached to the support at different positions. Using a vancomycin CSP similar to commercial Chirobiotic V, Dermaux et al. [29] baseline-separated warfarin and hexobarbital enantiomers in the RP mode with a column efficiency in the range of 30 000–40 000 plates/m, which is about 30% higher than that observed in corresponding HPLC separations.

Wikström et al. immobilized vancomycin in situ on prepacked diol-modified 5 μm 100 Å and 10 μm 1000 Å silica beads. They reported better enantioselectivity in the polar organic mode, in which they were able to separate a broader variety of chiral compounds. For example, basic compounds such as the β-adrenoceptor antagonist metoprolol were separated with efficiencies of 40 000–120 000 plates/m (Fig. 3).

Tryptophan enantiomers have been separated under RP conditions within 4 min with an efficiency of 35 000 plates/m using a capillary column packed with commercial 5 μm teicoplanin-bonded CSP

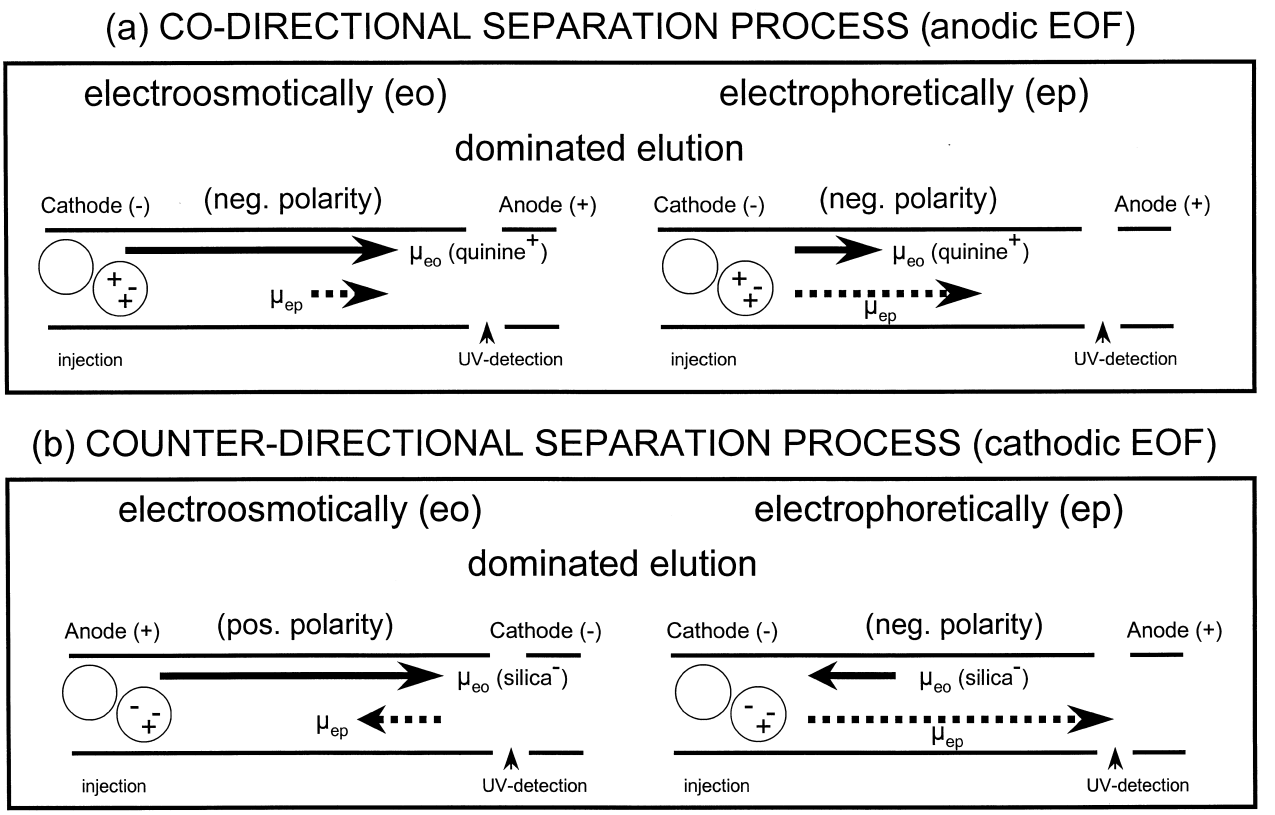

Fig. 4. Possible elution modes using amphoteric quinine carbamate-based CSPs and oppositely charged analytes.

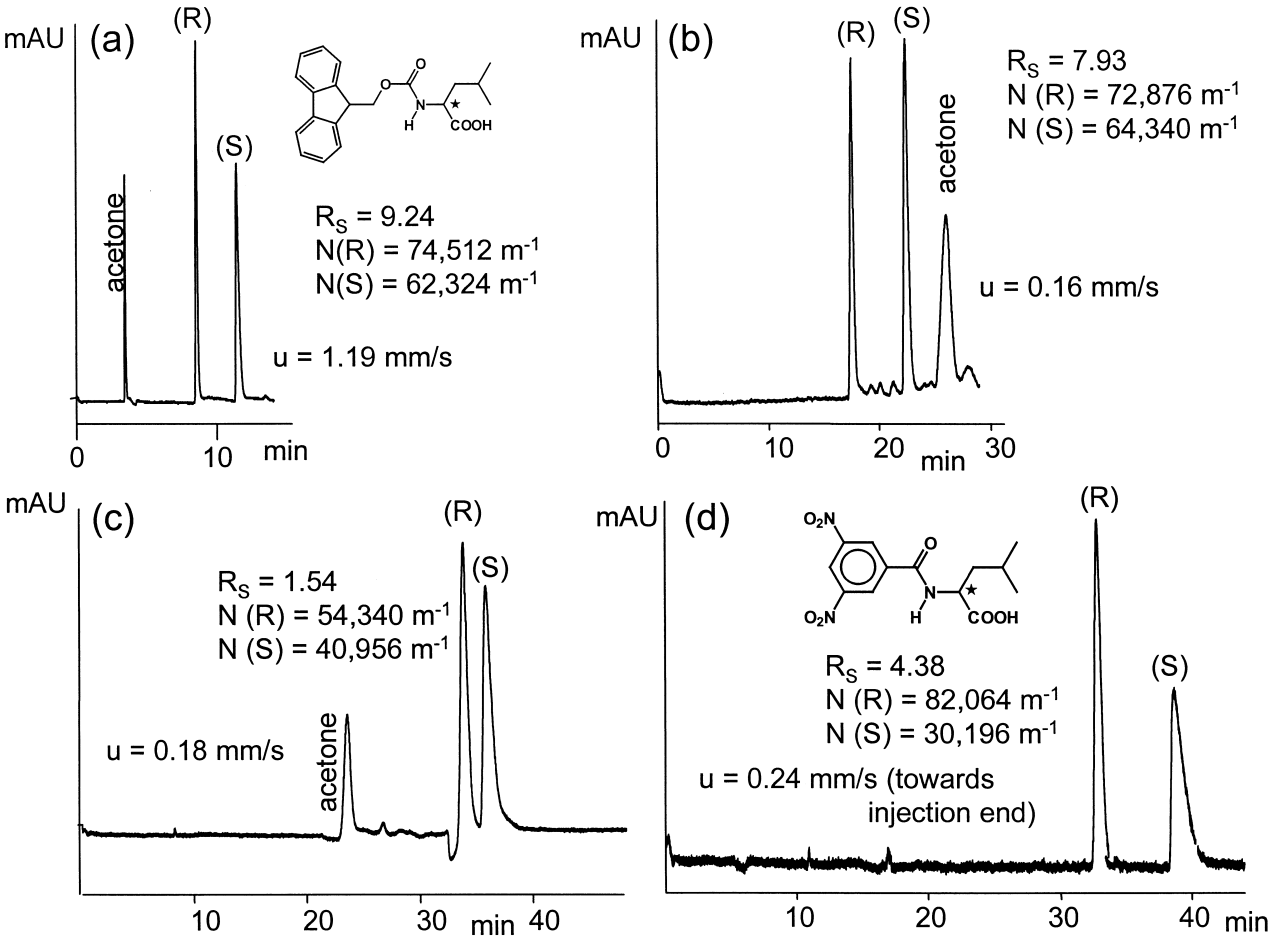

Fig. 5. Co-directional separations of (*R*,*S*)-Fmoc-leucine (anodic EOF) (a,b) and counter-directional separations of (*R*,*S*)-DNB-leucine (cathodic EOF) (c,d) on quinine-derived chiral anion exchangers based on different types of silica beads as chromatographic support: (a) elution of analytes driven by electroosmotic transport with minor electrophoretic migration contribution (co-directional electroosmotically dominated separation process). (b) Significant electrophoretic migration contribution results in elution of the analyte before the EOF marker (co-directional electrophoretically dominated separation process). (c) Counter-directional electroosmotically dominated separation process and (d) counter-directional electrophoretically dominated separation process. Conditions: capillary: 335 mm × 0.1 mm i.d. (250 mm effective length), CSPs: *O*-(*tert*-butyl carbamoyl) quinine immobilized onto thiopropyl-modified porous 3 μm Hypersil 120 (a,b) and non-porous 1.5 μm Micra NPS (c,d) silica beads. Separation conditions: (a) 0.2 mol/l acetic acid and 4 mmol/l triethylamine in acetonitrile–methanol (80:20), *T*: 20°C, −25 kV (−3.6 μA); (b) 0.2 mol/l acetic acid and 10 mmol/l triethylamine in acetonitrile–methanol (80:20), *T*: 20°C, −25 kV (−16 μA); (c) acetonitrile–0.1 mol/l MES (80:20), pH_a 6.0 (adjusted with triethylamine), *T*: 20°C, +6 kV (+0.37 μA); (d) methanol–0.1 mol/l MES (80:20), pH_a 6.0 (adjusted with triethylamine), *T*: 20°C, −15 kV (−5 μA); (reprinted with modifications from [32,33]. © 2000 Elsevier).

Chirobiotic T [25]. However, these studies also indicated limited stability of the CEC system in the RP mode and a narrower spectrum of compounds that could be separated compared to the application of identical CSP in HPLC. It is likely that the less defined and structurally inhomogeneous surface with a variety of ionizable functionalities having p*K* values close to each other contributes to difficulties encountered with these CSPs in CEC.

5.2.3. Chiral anion exchangers

In yet another approach, *O*-(*tert*-butyl carbamoyl) quinine selector was immobilized onto silica and used in its protonated form as a chiral anion exchanger for the CEC separation of chiral acids [31–33]. The ion–ion interactions are the primary driving force for SO–SA complex formation in this separation system. As discussed earlier, the ionic solute–sorbent interactions are thought to be less

suitable for CEC, since they are too strong to be counterbalanced by the low ion concentrations of mobile phases typically used in CEC to ensure high EOF. As a result, the analytes may not elute from the column. The solution to this problem can be sought in the use of non-aqueous mobile phases containing high concentrations of organic couterions as buffers and electrolytes. For example, solutions of acetic acid in acetonitrile–methanol with up to 600 mmol/l acetic acid were tested as a mobile phase in CEC without observing difficulties with Joule heat and bubble formation, since the current in these non-aqueous phases was significantly lower than in aqueous media [33]. This has led to faster separations, higher efficiencies, and better peak symmetries than in the aqueous RP mode.

Obviously, these silica-based chiral anion exchangers have an amphoteric character, since they contain both potentially positively charged quinine carbamate and negatively charged silanols. Accordingly, flow in either of the two possible directions can be observed depending on the pH of the mobile phase: cathodic EOF at pH higher than the apparent p*I* of the CSP, and anodic EOF at lower values. The apparent p*I* of this CSP depends on the type of silica used and on the extent of surface modification. Due to the primary ion exchange mechanism of these CSPs, suitable enantioselectivity can only be obtained for negatively charged analytes. Therefore, the contribution of electrophoretic migration of the analytes in this system must also be considered. This leads to a complex and delicate interplay of EOF, electrophoretic migration, and chromatographic retention. As a result, transport of solutes by EOF and electrophoretic mobility can be co- or counter-directional leading to four elution modes shown in Fig. 4. These separation modes were experimentally implemented by varying silica supports and mobile phase conditions (Fig. 5).

The immobilization of the basic *O*-(*tert*-butyl carbamoyl) quinine selector onto porous silica particles with large surface areas was very efficient leading to CSPs with high selector-loadings and apparently low contents of residual silanol functionalities [33]. Such CSPs afford anodic EOF at the typical working pH range below 6.5 both under aqueous RP and non-aqueous polar organic phase conditions (Fig. 4a). Changing pH, ionic strength, and solvent system, the relative contributions of electrophoretic and electroosmotic transport can be tuned. For example, electrophoretic migration at low pH and low ionic strength is negligible compared to transport by EOF (co-directional electroosmotically dominated separation; Fig. 5a). In contrast, the contribution of electrophoretic migration is dominant at higher pH after the addition of base, which also increases the ionic strength of the mobile phase, and concomitantly leads to a decrease in EOF velocity. Therefore, the EOF marker elutes later than the analytes that are well separated (co-directional electrophoretically dominated separation; Fig. 5b).

Quinine carbamate type CSPs with a low selector-loading and a high activity of silanol groups are obtained by immobilization of the selector on nonporous silica particles [32]. These amphoteric CSPs with a low p*I* afford cathodic EOF within the typical working pH range resulting from the negative net charge of the CSP under these conditions. Also such counter-directional separation processes (Fig. 4b) enable good enantioseparation of chiral acids. Elution of the negatively charged analytes in the positive polarity mode is observed under RP conditions at which electroosmotic transport dominates (counter-directional electroosmotically dominated separation; Fig. 5c). Changing the mobile phase by replacing acetonitrile as organic modifier with methanol leads to electrophoretically dominated elution in the negative polarity mode, while the EOF is directed towards the injection end of the capillary (counter-directional electrophoretically dominated separation process; Fig. 5d). These examples demonstrate that subtle changes in both mobile and stationary phase parameters can yield significant changes in the separation mechanism. Unlike the additive mode (vide infra), the elution order of the enantiomers is always determined by the relative intrinsic affinities of the enantiomers to the chiral selector moieties of the CSP.

5.2.4. Cyclodextrins

The EOF characteristics of CSPs based on neutral cyclodextrin moieties are less complex than those of the selectors with ionizable functionalities. Owing to their popularity and good selectivity, even in aqueous mobile phases, capillary columns packed with cyclodextrin-bonded or modified silica particles have also been evaluated in CEC. These CSPs are useful for the separation of chiral compounds bearing aromatic or other hydrophobic groups. In the RP mode, these analytes form inclusion complexes within the hydrophobic cavity of the cyclodextrin selector, occasionally accompa-

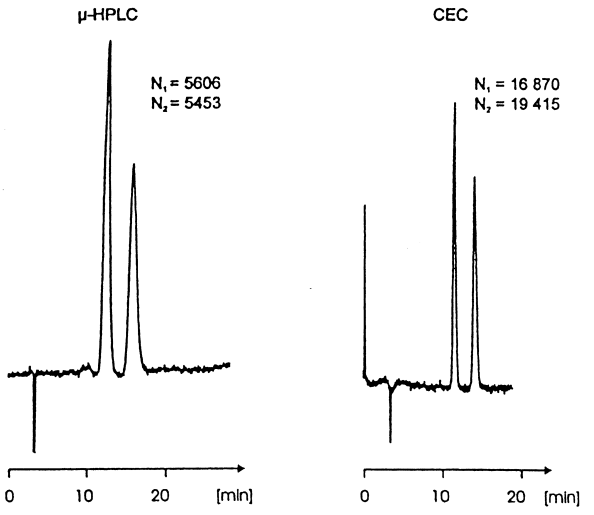

Fig. 6. Capillary HPLC and CEC enantioseparations of mephobarbital. Conditions: 23.5 cm (total length 40 cm) × 100 μm i.d. capillary packed with permethyl-β-cyclodextrin-bonded silica; phosphate buffer (5 mmol/l, pH 7.0)–methanol (4:1, v/v); CEC, +20 kV; 10 bar; μLC, 140 bar (reprinted with permission from [35]. © 1998 Elsevier).

nied by interaction with polar and/or hydrogen bonding functionalities at the hydrophilic outside upper and lower rims. The stability of the complex is determined by both the size and hydrophobicity of the binding groups forming the inclusion complex, and the strength of interaction can be controlled by the percentage of organic modifier in the aqueous mobile phase. In contrast, the chiral discrimination in the polar organic mode is thought to primarily rely on stereoselective interactions at the hydrophilic rims dominated by hydrogen bonding. However, CEC enantioseparations on cyclodextrin-based CSPs in this mode have yet to be demonstrated.

Enantiomers of benzoin, hexobarbital, and both dansyl and 2,4-dinitrophenyl derivatives of amino acids could be separated on capillary columns packed with CSP containing native β-cyclodextrin functionalities (5 μm Cyclobond I) [34]. Substitution of potassium phosphate with triethylammonium acetate buffer led to reversal of EOF and facilitated the co-directional separation of acidic compounds [34]. This reversal, observed with mobile phases containing only low percentages of methanol (e.g. not exceeding 15%), can be explained by adsorption of triethylammonium ions onto the surface of the stationary phase and formation of a dynamic anion exchanger.

The separation of enantiomers of neutral chlorthalidone and basic mianserin on hydroxypropyl β-cyclodextrin-bonded phase (commercial 5 μm Cyclobond I 2000 RSP) was reported by Lelièvre et al. [10].

Wistuba et al. [35] used 300 Å pore size 5 μm permethyl-β-cyclodextrin-bonded silica beads. They separated several barbiturates, benzoin, glutethimide, methylthiohydantoin-proline, and methyl mandelate with efficiencies ranging from 20 000 to 40 000 plates/m. Direct comparison of μHPLC and CEC separations of mephobarbital on the same capillary column showed that using the latter technique resulted in three times higher column efficiencies and an increase of resolution from 2.08 to 3.39 without any change of enantioselectivity (Fig. 6).

Wide-pore silica beads (300 Å) coated with permethylated cyclodextrin covalently linked via an octamethylene spacer to dimethylpolysiloxane (Chirasil-Dex) led to further improvement in the column efficiency [36]. Since the coating effectively shields the residual silanol functionalities of CSP, 20% of bare silica beads were admixed to the chiral packing to increase EOF by 26%. This study demonstrated the separation of the same analytes

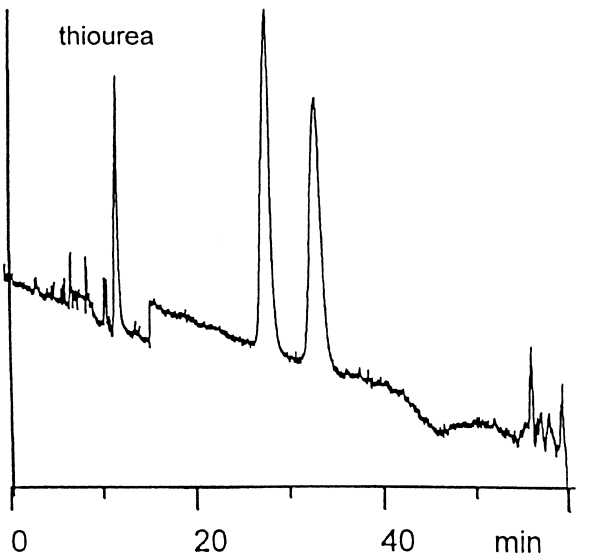

Fig. 7. CEC enantioseparation of indapamide on cellulose tris(3,5-dimethylphenylcarbamate) CSP (7 μm, 4000 Å). Conditions: capillary dimension: 20 cm packed length × 100 μm i.d., mobile phase: acetonitrile–5 mmol/l phosphate buffer (pH 7) (50:50 v/v), 20°C, voltage 20 kV (reprinted with permission from [38]. © 2000 Elsevier).

(barbiturates, benzoin, glutethimide, methylthiohydantoin-proline, and methyl mandelate) and also of herbicides based on esters of aryloxycarboxylic acid. In comparison to the covalently bonded permethyl-β-cyclodextrin CSP analog, slightly higher efficiencies of 30 000–70 000 plates/m were observed for this CSP. The hydrophobic microenvironment and shielding of the polar silanol groups with the polysiloxane film improves enantioselectivity. For example, 1-(2-naphthyl)-ethanol could be separated only on the Chirasil-Dex coated CSP.

5.2.5. Modified polysaccharides

CEC enantiomer separations using the most popular class of chiral HPLC packings, the cellulose-based CSPs, have only recently been demonstrated [37,38]. Krause et al. reported the pressure-assisted CEC separation of bendroflumethiazide and indapamide on a CSP obtained by coating wide-pore (1000 Å) 3-aminopropylsilyl functionalized 5 μm silica beads with tris(3,5-dimethylphenylcarbamoyl)cellulose [37]. The amine functionalities that were not completely covered by the coating affected the anodic EOF. Recently, Mayer et al. presented a more detailed study about the behavior of polysaccharide CSPs in CEC [38]. Using a new chemically more stable cellulose phase prepared by coating cellulose tris(3,5-dimethylphenylcarbamate) onto 5 or 7 μm macroporous silica beads (pore size 4000 Å) followed by crosslinking, various basic and neutral compounds were separated including lorazepam, benzoin, indapamide (Fig. 7), *trans*-stilbene oxide, 1-(α-naphthyl)ethanol, and glutethimide with efficiencies in the order of 20 000–50 000 plates/m [38]. The EOF velocities were low, typically less than 1 mm/s, resulting from a more effective coating of the silanol groups. Very long run times have been the consequence. Acceleration of the separation was achieved by using packed segments of only 8 cm in length. A direct comparison of the nano-HPLC and CEC separations using the same capillary confirmed the 3–4 times higher efficiency of the electroosmotically driven separation mode without any changes in selectivity factors.

5.2.6. Synthetic polymer phases

Typical normal-phase chiral HPLC packings, poly(*N*-acryloyl-(*S*)-phenylalanine ethylester) covalently attached to silica beads (ChiraSpher®) [37], and wide-pore (1000 Å) aminopropyl-modified 5 μm silica beads coated with helically chiral poly(diphenyl-2-pyridylmethyl methacrylate) [39] were applied for both aqueous and non-aqueous CEC using methanol as the mobile phase and ammonium acetate as the electrolyte. Krause illustrated in the latter study that pressure-assisted CEC significantly reduces run times compared to the solely pressure or electrically driven separations. Benzoin derivatives, Tröger's base, *trans*-stilbene oxide are examples of separated racemates.

5.2.7. 'Brush' phases

The highest column efficiencies for silica-based chiral packings were reported by Wolf et al. [40,41]. On two Pirkle type CSPs functionalized with low-molecular selectors, (*S*)-Naproxen amide CSP and (3*R*,4*S*)-Whelk-O1 CSP based on 100 Å narrow pore size 3 μm silica beads, efficiencies ranging from 100 000 to 200 000 plates/m were obtained for a variety of neutral enantiomers, mostly *N*-3,5-dinitrobenzoyl derivatives of amino acid esters and amides. These high efficiencies can be ascribed to both the small diameter of beads, the favorable

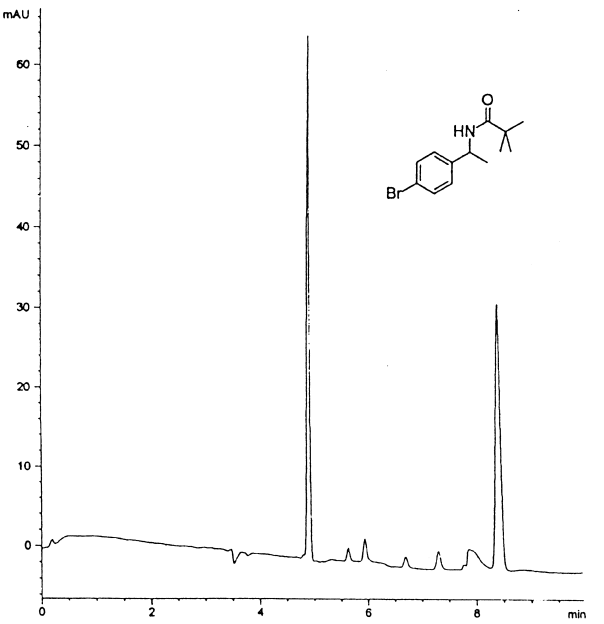

Fig. 8. Enantioseparation of *N*-pivaloyl-1-(4-bromophenyl)-ethylamine on 3 μm (3*R*,4*S*)-Whelk-O1 CSP. Conditions: MES (25 mmol/l, pH 6.0)–acetonitrile (1:3.5); 25 kV. 39.3 total capillary length; 30.5 cm effective length (reprinted with permission from [40]. © 1997 Elsevier).

mass transfer kinetics, and the flat EOF profile (Fig. 8).

While several of the CSPs discussed above operate primarily in aqueous mobile phases that are amenable to typical CEC, other CSPs functionalized with selectors such as the modified cellulose, synthetic polymers, and Pirkle type ligands are known to afford higher enantioselectivity in the normal-phase mode. Despite these limitations, also these CSPs could be successfully employed in CEC. However, only a significantly narrower spectrum of compounds could be separated as compared to standard HPLC. A partial solution to this problem is the use of non-aqueous polar organic mobile phases, in which the required electrolytes can readily be dissolved [12,30,33,39]. On the other hand, the feasibility of CEC using mixtures of *n*-hexane with alcohols or acetonitrile containing ammonium or triethylammonium acetate as electrolytes has also been demonstrated [42,43]. Such conditions could expand the scope of applicability of typical normal-phase packings in CEC.

The results clearly document that typical chiral HPLC packings can be successfully used for enantiomer separations in CEC and higher column efficiencies close to those predicted by theory can often be achieved. However, the well-known bubble formation at the transition from the end frit to the open section of the capillary column remains still a problem. Therefore, pressures of 0.3–1.2 MPa at both ends of the capillary must be applied to prevent this undesirable effect.

5.3. Monolithic columns

Although the preparation of packed capillary columns appears to be well established for the manufacture of CEC columns, their reproducible production remains difficult. The preparation requires several steps, among them packing of the beads into the capillary tubing and frit fabrication. These difficulties in packing technology triggered the search for alternative approaches to the preparation of capillary columns. Columns containing monolithic packings are one of these new technologies that have already proven their usefulness in CEC. These monoliths do not require fabrication of retaining frits. The lack of frits represents one of the major advantages of this class of capillary columns. These columns consist of a single piece of continuous stationary phase, which may be covalently anchored to the capillary wall. The monolithic material is traversed with numerous throughpores that are sufficiently large to allow flow of the mobile phase. A number of different approaches to monolithic stationary phases for CEC have already been proposed [44], and some of them were also used for the preparation of enantioselective columns.

A method representing a 'hybrid' between packed and monolithic columns has been proposed by Lin and co-workers. Molecularly imprinted polymers were prepared by a typical non-covalent imprinting technique using various amino acids as templates [45–48]. These bulk polymers were ground, sieved, and the less than 10 μm small irregular particles were packed into capillary tubing. Finally, the particulate packed bed was entrapped into a polyacrylamide gel. Although some resolution of enantiomers of the imprinted molecule was achieved, the peak shape was rather poor.

Using a similar concept, Chirica and Remcho entrapped irregular particles of a polymer, molecularly imprinted with *N*-dansyl (*S*)-phenylalanine, in a silicate matrix [49]. The column was first packed with imprinted polymer particles, flushed with a potassium silicate solution, and then gradually heated from 40 to 160°C over a period of several days to form the silicate matrix. The advantage of this approach is that the silicate matrix also provides a sufficient number of charged groups required for strong EOF that are not present in the imprinted polymer itself. In comparison to the HPLC mode with the same packing, both higher efficiency and much faster separation of *N*-dansyl phenylalanine enantiomers were achieved using CEC.

The above described embedding approaches that require the out-of-column preparation of the particles with the desired chromatographic properties, their packing, and subsequent embedding to form the monolithic matrix are complex and tedious. More straightforward is the direct preparation of chiral monolithic separation media with the desired chromatographic properties within fused-silica tubing by in situ polymerization. The major advantage of the direct preparation approach is its simplicity. The capillary is filled with a homogeneous polymerization mixture and the in situ polymerization process initiated. This method enables parallel synthesis of large numbers of capillaries with the desired length, or alternatively, long capillaries can be prepared and cut to any length. More-

over, the preparation of multiple columns simultaneously from the same polymerization mixture helps to improve the column-to-column reproducibility.

Nilsson [50] and Lin [51] demonstrated the in situ preparation of porous molecularly imprinted chiral monoliths within vinylized fused-silica capillaries using respectively photopolymerization and thermally initiated polymerization processes. There are two major factors that affect performance of the imprinted monoliths: (i) the number and fidelity of the chiral cavities created by the imprinting process, and (ii) the porous structure of the monolith. The former requires a choice of suitable functional monomers to obtain imprinted binding sites with well defined shape and properly arranged functionalities that are a result of well defined self-organized complexes between functional monomers and template molecule during the polymerization process. Methacrylic acid, trifluoromethacrylic acid, vinyl pyridines and methacrylamide are frequently used functional monomers. All of these monomers may interact with functionalities of the template via hydrogen donor–acceptor and/or Coulombic interactions. A high percentage of crosslinking monomers is typically used to ensure structural rigidity of the imprinted materials. Trimethylolpropane trimethacrylate, pentaerythritol triacrylate, and pentaerythritol tetraacrylate are used more often than ethylene dimethacrylate. The driving force for this preference is the perception that a lower percentage of the polyvinyl crosslinkers may lead to the same degree of crosslinking. A lower percentage of crosslinker, in turn, allows the use of higher contents of functional monomers. As a consequence, a higher density of imprinted sites can be created in the polymer that is expected to provide higher loading capacity, increased enantioselectivity and improved resolution. Polymerizations at low temperatures (e.g. −20°C) appear to afford better defined chiral cavities since the association complexes of functional monomers and template molecule are more stable. In this case, the polymerization is started by photoinitiation.

Two different approaches were used to generate the porous structure suitable for the flow-through application in CEC: (i) addition of porogenic solvents to the monomer mixture [52] and (ii) termination of the polymerization reaction before its completion [50]. The latter requires both perfect timing and absolute control of reaction conditions. Therefore, the former approach appears to be more reproducible. Suitable porogens are aprotic solvents that do not interfere with the non-covalent binding between complementary functionalities. Nilsson and co-workers used toluene with 1–25% isooctane in combination with UV-initiated polymerization. They obtained so-called 'superporous' chiral monolithic capillary columns well suited for the enantioseparation of the racemic template in CEC mode. For example, β-adrenergic antagonists and local anesthetics were rapidly separated [50,52–54]. Closely related structural analogs of the imprint molecules were also separated as a result of cross-selectivity of this CSP.

Lin et al. used a mixture of ammonium acetate and chloroform as the porogen in combination with thermal initiation. Since this binary porogen was immiscible, homogeneization with the other components of the polymerization mixture was induced by sonication. An (*S*)-phenylalanine anilide imprinted monolithic column separated phenylalanine enantiomers, and some separation was also observed for racemates of tyrosine and phenylglycine [51].

Even though electrically driven flow significantly enhances column efficiencies compared to HPLC separations on MIPs using the pressurized flow, and the 'superporous' imprinted monoliths prepared by UV polymerization afforded quite good peak shapes, high efficiencies could be obtained also with the monolithic MIPs only for the less retained enantiomer. Therefore, other approaches to the preparation of chiral monolithic columns are desirable. One of those is the direct incorporation of functional monomers with attached selector moieties into the monolith by a copolymerization.

A very simple technique for the preparation of polymethacrylate-based monoliths that was introduced in the early 1990s for HPLC [55] has also been adapted for the preparation of chiral 'molded' rigid monolithic capillary columns [56–58]. This single-step approach includes in situ copolymerization of a mixture of methacrylates consisting of a chiral monomer, comonomer(s), and a crosslinker within the confines of an untreated fused-silica capillary. The polymerization mixture also involves porogenic solvents, and the polymerization is initiated either thermally or by UV light. Due to the rigidity of the resulting macroporous monoliths, its anchoring to the capillary wall may not be required.

Optimal porous structure of these copolymerized monoliths is of paramount importance to

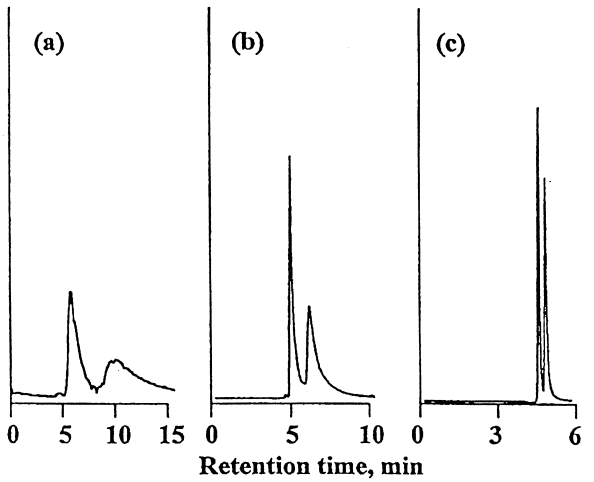

Fig. 9. Effect of hydrophilicity of chiral monolithic columns on the CEC separation of *N*-(3,5-dinitrobenzoyl)leucine diallylamide enantiomers. Stationary phase, copolymer of (*N*-[(2-methacryloyloxyethyl)oxycarbonyl]-(*S*)-valine-3,5-dimethylanilide) with ethylene dimethacrylate and butyl methacrylate (a), glycidyl methacrylate (b), and hydrolyzed glycidyl methacrylate (c). Column dimension, 100 μm i.d. × 30 cm active length; mobile phase, 80:20 v/v acetonitrile–5 mmol/l phosphate buffer pH 7; voltage 25 kV; pressurization: 2 bar inlet and outlet end (reprinted with permission from [44]. © 2000 Wiley-VCH).

achieve both good flow properties and high column efficiencies. The porous properties can be well controlled by changes in the composition of binary or ternary porogenic solvent mixtures. We found that EOF velocities are not only determined by the surface charge density but also by the pore size. Although the current theory of CEC predicts independence of the EOF on the diameter of the flow channels, the linear flow velocity was found to be directly proportional to the pore diameter of the monoliths. The larger the through-pores, the higher the flow velocity. This fact was explained by the lower tortuosity of the larger pore monoliths that enable shorter flow path and correspondingly higher linear flow rates. The best CEC performance, on the other hand, was observed for monoliths with pore diameters ranging from 400 to 700 nm as measured by mercury intrusion porosimetry in the dry state.

In addition to variability in the design of the porous structure, monolithic technology also allows tailoring of the surface chemistry. Various ligands or selectors required for both specific chromatographic applications and control of surface charge density and polarity can easily be introduced into the monolithic structure. For example, the EOF direction is easily controlled by copolymerization of either positively or negatively chargeable monomers. Similarly, optimizing the surface chemistry using comonomers with favorable functionalities can reduce the non-specific interactions. Obviously, a careful tuning of properties is required to obtain highly efficient and selective monoliths best suited for the desired separation.

We incorporated 'brush type' chiral selector containing monomer *N*-[(2-methacryloyloxyethyl)oxycarbonyl]-(*S*)-valine-3,5-dimethylanilide directly into a macroporous monolith [56]. Since this monomer does not contain chargeable functionalities, 2-acrylamido-2-methylpropanesulfonic acid was added to the polymerization mixture to afford negative surface charge over the entire pH range. Only 0.3 wt% of this monomer was sufficient to achieve high EOF. The resulting chiral monoliths exhibited enantioselectivity for the separation of (*R*)- and (*S*)-*N*-(3,5-dinitrobenzoyl)leucine diallylamide under RP conditions. In order to minimize the non-specific interactions that are detrimental to both enantioselectivity and efficiency, the polarity of the monolith surface was stepwise increased. Very poor efficiency was observed for monolithic columns prepared from the chiral monomer and ethylene dimethacrylate to which butyl methacrylate was added as a comonomer (Fig. 9a). In contrast, monoliths with more polar lateral epoxypropyl functionalities prepared by substitution of butyl methacrylate with glycidyl methacrylate led to columns with higher efficiency (Fig. 9b). However, a dramatic improvement was achieved after hydrolysis of the epoxides to diols. A column efficiency of 60 000 plates/m was found for the separation of *N*-(3,5-dinitrobenzoyl)leucine diallylamide enantiomers using this monolith (Fig. 9c).

Recently, we published another example that demonstrates the ease of control of surface chemistry of monoliths prepared by copolymerization [57,58]. Enantioselective monolithic CEC columns were prepared using an ionizable chiral monomer, *O*-[2-(methacryloyloxyethyl)carbamoyl]-10,11-dihydroquinidine. As described above, this selector operates in an anion exchange retention mechanism. Advantageously, the ionizable chiral monomer eliminates the need for the additional chargeable comonomer that had to be used in our previous study. The quinidine moieties that are positively charged under CEC conditions afford

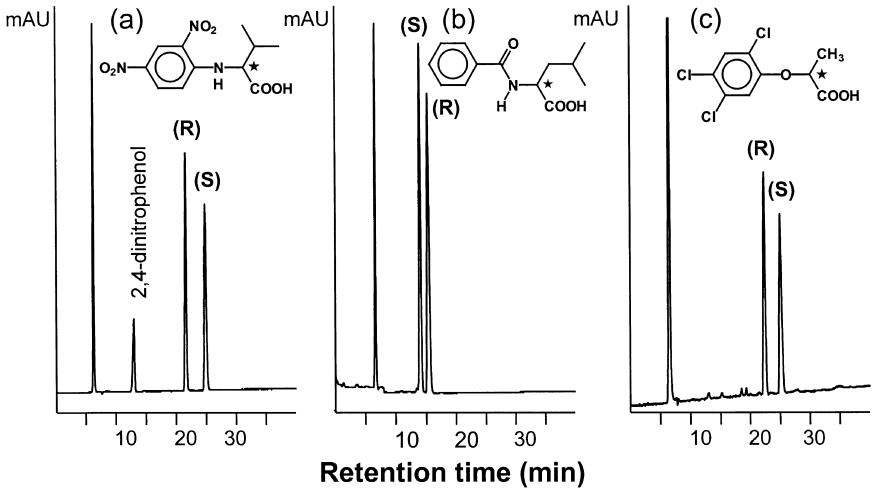

Fig. 10. CEC separations of the enantiomers of *N*-2,4-dinitrophenyl valine (a), *N*-benzoyl leucine (b), and 2-(2,4,5-trichlorophenoxy)propionic acid (Fenoprop) (c) on a 15 cm long quinidine-functionalized chiral monolith. Conditions: polymerization mixture, chiral monomer 8 wt%, 2-hydroxyethyl methacrylate 28 wt%, ethylene dimethacrylate 4 wt%, 1-dodecanol 30 wt%, and cyclohexanol 30 wt%, UV-initiated polymerization for 16 h at room temperature, pore diameter 1097 nm, capillary column 335 mm (250 mm active length) × 0.1 mm i.d., EOF marker acetone, mobile phase 0.6 mol/l acetic acid and 6 mmol/l triethylamine in 80:20 mixture of acetonitrile and methanol, separation temperature 50°C, voltage −25 kV (reprinted with permission from [58]. © 2000 American Chemical Society).

both selectivity due to stereoselective interaction with enantiomers and simultaneously anodic EOF. Using hydrophilic 2-hydroxyethyl methacrylate as a comonomer, monoliths with highly hydrophilic surfaces were directly prepared. Rather surprisingly, monoliths comprising 10 and 20 wt% of ethylene dimethacrylate exhibited better mass transfer characteristics and thus higher column efficiencies compared to those with a typical 40 wt% crosslinking despite the equal 'dry state' pore size adjusted to 1000 nm. This was attributed to swelling effects and to higher homogeneity of the solid–liquid interface. Since the less crosslinked materials swell and the overall space available within the column is fixed, the pores are partly filled with the swollen polymer chains. This process then improves the chromatographic properties of the monolith. However, this decrease in the pore size also leads to a concomitant decrease in the flow velocity.

Efficiencies reaching levels typically reserved for CE could be obtained with these quinidine-functionalized monolithic columns that separate acidic chiral compounds in both aqueous RP and non-aqueous polar organic phase modes (Fig. 10). For example, column efficiencies of 242 000 and 194 000 plates/m were obtained for the separation of *N*-2,4-dinitrophenyl valine enantiomers using a 15 cm long optimized chiral monolith (Fig. 10a). Similarly high efficiencies were also obtained for other chiral acids such as *N*-benzoyl leucine (Fig. 10b) and α-aryloxycarboxylic acid herbicide Fenoprop (Fig. 10c) [58]. Moreover, due to high enantioselectivity and resolution, monolithic segments only 8.5 cm long proved to be sufficient for baseline separations of a wide variety of chiral acids (Fig. 11). This decrease in length also enabled substantial acceleration of the CEC separations.

In yet another approach, charged low crosslinked polyacrylamide gels with β-cyclodextrin moieties physically incorporated or covalently attached to the polymer were prepared within capillaries pre-functionalized with methacrylate groups [59–61]. Typically, the complex aqueous polymerization mixture contains only 5–10% total monomers. The crosslinker accounts for 5–10% of the total monomers. Therefore, the hydrophilic polyacrylamide matrix is highly swollen in water. Both negatively and positively charged gels were prepared that afforded either cathodic or anodic EOF. Advantageously, these monoliths allow the separation in mobile phases that do not contain organic modifier. The organic solvent would interfere with the binding of aromatic groups within the hydrophobic β-cyclodextrin cavity and would

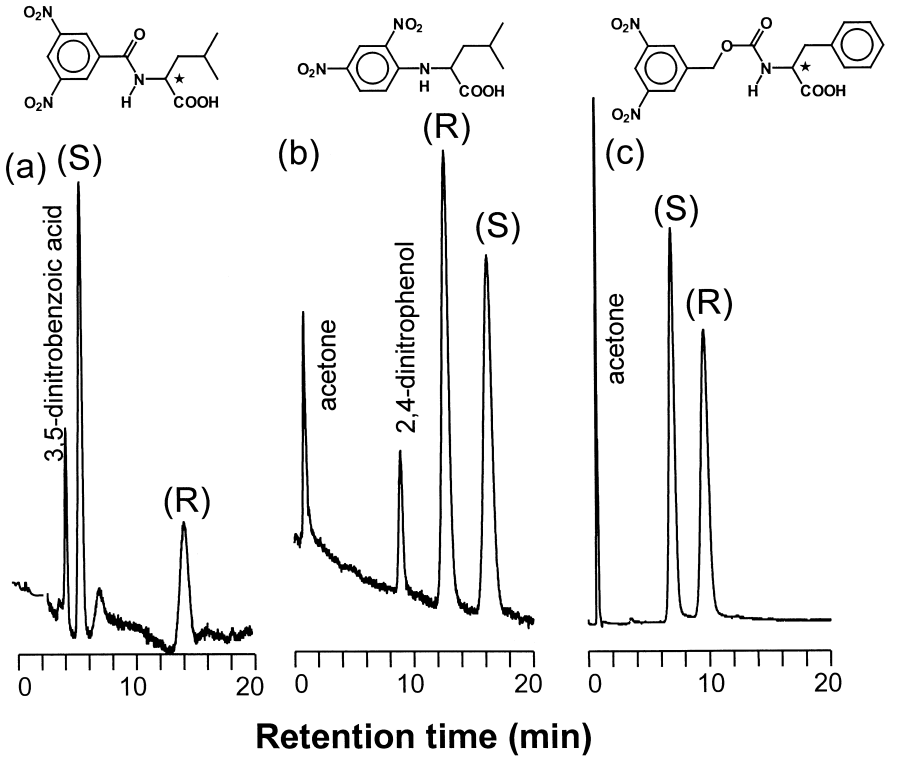

Fig. 11. Separation of the enantiomers of (a) *N*-3,5-dinitrobenzoyl leucine, (b) *N*-2,4-dinitrophenyl leucine, and (c) *N*-3,5-dinitrobenzyloxycarbonyl phenylalanine on quinidine-functionalized chiral monoliths with bed length of 8.5 cm by short-end injection. Capillary dimension: 100 μm i.d., total length: 33.5 cm; polymerization mixture: 12 wt% chiral monomer, 20 wt% 2-hydroxyethyl methacrylate, 8 wt% ethylene dimethacrylate, 50 wt% 1-dodecanol, and 10 wt% cyclohexanol. UV-initiated polymerization for 16 h at room temperature, pore diameter 1317 nm, EOF marker acetone, mobile phase 0.4 mol / l acetic acid and 4 mmol / l triethylamine in 80:20 mixture of acetonitrile and methanol, separation temperature 50°C, voltage +30 kV, injection at +15 kV for 5 s.

decrease enantioselectivity. The separations of cationic terbutaline and propranolol as well as neutral benzoin were demonstrated using a negatively charged gel-filled capillary [59,60]. In their recent study, this group also separated dansylated amino acids and some other acidic compounds on positively charged polyacrylamide gels with covalently attached allylcarbamoylated β-cyclodextrin. The column efficiencies claimed in this work reached up to 150 000 plates / m. With selectivity factors of 1.0–1.2, resolutions of up to 7 could be obtained [61].

6. Conclusion

The large number of studies published to date document the potential and feasibility of enantioseparation by CEC. Clearly, the electroosmotically driven flow of CEC affords higher column efficiencies compared to the pressure-driven HPLC separations. On the other hand, if electrophoretic migration plays a significant role in the separation process, an exact measure for enantioselective molecular recognition and chromatographic enantioselectivity, respectively, cannot be derived directly from the calculated separation factor of the electrochromatogram obtained by CEC. One should be aware that in CEC of charged compounds, apparent enantioselectivity factors are obtained (as in CE), unless correction for the electrophoretic process is performed. As a consequence, enantioselective HPLC may offer some advantages in chiral recognition studies, since calculated separation factors are directly correlated to chromatographic $\Delta\Delta G$ values. On the other hand, for stereoselective bioanalytical measurements such as protein–ligand or other SO–SA binding

studies electrophoretic methods may be preferred due to flexible incorporation of various SOs to the background electrolyte and simple variation of SO concentrations. Compared to CE and MEKC enantioseparation methods, the main advantages of CEC are more flexibility in the choice of running electrolyte solutions, since selector solubility in the background electrolyte is no longer a prerequisite, and better feasibility of MS detection. Racemates of all kind, i.e. of neutral, basic, and acidic chiral analytes, were separated by CEC methods. For charged analytes, however, the effect of electrophoretic migration contribution on retention time, enantioselectivity, and column efficiency remains to be studied and deconvoluted.

The variety of current approaches ranges from a simple addition of the chiral selector to the mobile phase in combination with a simple RP CEC to the more elegant separation systems that include enantioselective OT, packed, and monolithic columns. It is likely that techniques involving OT columns may not be able to compete so easily with the rapid development of both packed and monolithic capillary columns. The low sample-loading capacity of OT columns that easily leads to column overloading, with fatal effects on efficiency and resolution, may be problematic when applied for the determination of enantiomeric impurities in the range of 1% and less. In contrast, enantioselective CEC with packed and monolithic columns may become widely accepted in the future, not least due to the fact that these approaches are easily amenable to MS detection. The direct transfer of the typical HPLC column technology to the capillary format may currently appear straightforward taking into account the advantage of the long tradition in the use of conventional chiral packings in HPLC. However, the enantioselective monolithic columns are easier to prepare and may offer more flexibility in the design of desired surface chemistries. We expect that the use of monolithic columns will grow in the near future, and monoliths will be directly prepared using diverse chiral selectors. Similarly, it is very likely that the other approaches to monolithic columns such as the entrapment of conventional chiral packings into continuous matrices will be redesigned for enantioselective CEC. This will result in a number of alternative capillary columns and enantioselective composite materials. Moreover, many of the monolith technologies appear to be easily transferable to analytical microfluidic devices and chip technology.

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